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Fibrin deposition in the kidney in post-ischaemic renal damage

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Summary. To investigate whether fibrin deposition in the kidney occurs in ischaemic acute renal failure, rats were subjected to a left renal artery occlusion (RAO) for 1 h and contralateral nephrectomy. The animals were killed 0, 5, 15 and 60 min after re-establishment of the circulation. Kidney tissue was snap–frozen for immunofluorescence microscopy and scanning electron microscopy, and immersion-fixed for light microscopy and transmission electron microscopy. Immunofluorescence studies showed small amounts of immune reactive fibrinogen/fibrin deposits in the peritubular capillaries. Substantial amounts of fibrinogen/fibrin positive material was observed in Bowman's space and in the tubular lumina. Scanning electron microscopy of freeze–dried tissue disclosed granular and fibrillar material in Bowman's space. A temporary enlargement of Bowman's space was noted; it may have been caused by tubular obstruction. No deposits were found in the control (right) kidneys and in kidneys of heparinized rats subjected to RAO. The results indicate that fibrin deposition occurs in the ischaemic model of acute renal failure in rat, both intravascularly and in the urinary space. Its importance for the development of renal functional impairment remains to be studied.

Keywords: rat, renal artery occlusion, acute renal failure, fibrin deposition

Patients with disseminated intravascular coagulation often develop acute renal failure (ARF), and massive fibrin deposition is found in the renal glomeruli (Kincaid-Smith 1972). Minor fibrin deposition also occurs in most other cases of ARF, to judge from the results of electron microscopic studies of renal biopsies (Clarkson *et al.* 1970) and from the urinary excretion of high molecular weight fibrin degradation products in such patients (Larsson *et al.* 1971). It is not clear whether the fibrin is of pathogenetic importance. Experimentally, intravenous infusion of thrombin in combination with inhibition of fibrinolysis causes heavy deposition of fibrin in the renal glomeruli, resulting in ARF with an elevated urea nitrogen concentration and a markedly reduced glomerular filtration rate (Rammer 1973). After subcutaneous injection of glycerol causing ARF in rats, Wardle and Wright (1973) using immunofluorescence technique found substantial deposition of fibrin in the glomeruli. In the ischaemic model of ARF induced by

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renal artery occlusion (RAO) contradictory results have been obtained. Mason *et al.* (1984) and Frega *et al.* (1976), could show no fibrin in studies of immersion fixed material in rat, using light and transmission electron microscopic methods. Hansson (1983), on the other hand, who used transmission and scanning electron microscopy of perfusion-fixed tissue, found intravascular fibrin deposits in peritubular capillaries after RAO in rabbit. It is possible that differences in fixation and detection methods, in which it is difficult to preserve small amounts of fibrin, could explain the discrepancies in the results.

The present study was performed in order to determine whether fibrin deposition in the kidney occurs in rat after RAO. We have tried various preparatory techniques for light and electron microscopy and for immune histochemistry in efforts to improve the possibility of detecting fibrin deposits.

Material and methods

Animals. Twenty female Sprague-Dawley rats (Anticimex Farm, Stockholm, Sweden) weighing 200–250 g were used. They had free access to food (Ewos rat pellets) and tap water.

Experimental procedure. Anaesthesia was introduced by an intraperitoneal injection of Inactin (Byk-Gulden), 120 mg/kg body weight (b.w.). The animals were placed on a heating pad to keep the body temperature at 37 ± 1°C, and were tracheotomized. The two kidneys were exposed through a midline incision. The vessels of the right, control kidney were ligated and the kidney was removed. The left kidney and renal artery were freed from surrounding structures, and the artery was clamped with an artery forceps. The abdomen was then closed. After 60 min flow was re-established by removing the forceps. At the end of the experiment the left kidney was removed, cleaned, and hemisected. Groups of three rats were killed at 0, 5, 15, and 60 min after reflow. Five rats were given, 0.5 ml of Heparin (Vitrum) solution (2000 iu per kg b.w.) 5 min before RAO.

Immunohistochemistry. Small pieces from all kidneys were snap-frozen in a dry-ice-isopentane mixture, cryostat sectioned (4–5μm), air dried, and ethanol fixed. After fixation the sections were rapidly washed in phosphate buffered saline (PBS), and then incubated in FITC-conjugated monospecific goat anti-rat antibodies to fibrinogen and albumin (Nordic Immunologic Lab), respectively, both diluted 1:40 with PBS. The sections were rapidly washed twice in PBS, mounted in 20% glycerol-PBS, and examined with a Zeiss epi-illumination fluorescence microscope with filter combination for FITC excitation. The specimens were photographed with Kodak Plus-X pan film, exposure time 45–50 seconds (Zeiss Planapo 63/1.5 Oel).

Light microscopy. Several small pieces from all kidneys were fixed in formol-sublimate (Stieve's fluid) for 2 h and in 4% buffered formalin for 12 h. Dehydration, embedding in Paraplast, cutting with glass knives into 1 μm sections, and staining with periodic acid-silver methenamine (PASM) were performed as described by Eneström (1981). Sections were also stained for fibrin by a modified picro–Mallory method and with phosphotungstic acid haematoxylin (PTAH) (Disbrey & Rack 1978). The diameters of the capillary tufts and the whole glomeruli were measured with an occular micrometer. Twenty glomeruli were measured in each animal.

Scanning electron microscopy. Snap-frozen small pieces from the kidneys were either thawed in 2% glutaraldehyde in 0.1 M Na-cacodylate-HCl for 12 h, post-fixed in 1% osmium tetroxide, and dehydrated in graded alcohols, or were freeze-dried for 24 h (Tis-U-Dry TFD-130, FTS Systems). The kidney pieces were embedded in Epon without hardener for 12 h and then cracked in liquid nitrogen. The epoxy resin was removed by incubating the sections in propylene oxide. They were washed in absolute ethanol and

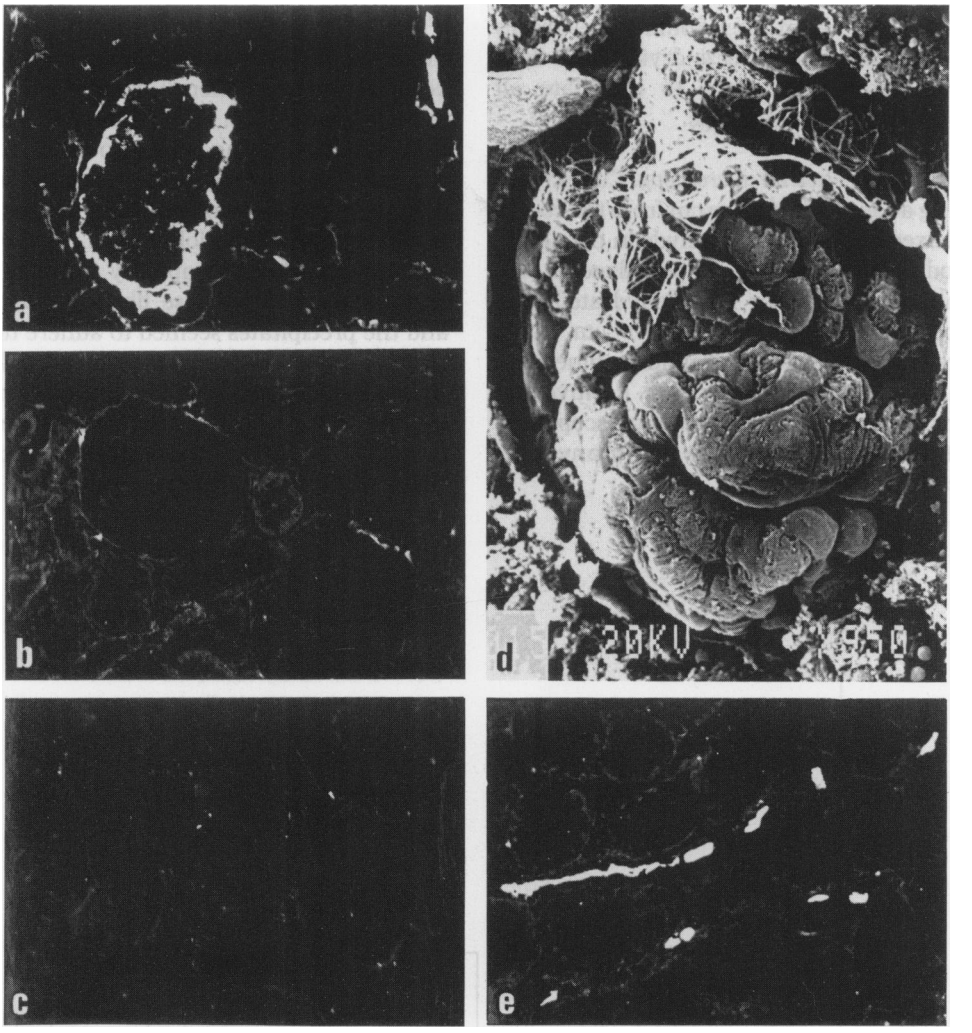

Fig. 1. Detection of fibrinogen/fibrin-related antigens in rat kidney after left renal artery occlusion (RAO) and re-establishment of the circulation for 15 min. ***a***, There is strong immune reactivity for the antigen, with dense fibrillary deposits in the urinary space and also between the capillaries of a glomerulus. Note also the deposits in tubular basement membrane adjacent to an intertubular vessel, with precipitates; direct immunofluorescence (dif), × 190. ***b***, No immune reactivity in the glomeruli of control kidney; (dif), × 190. ***c***, Injection of heparin (2000 iu/kg bw) 5 min before RAO eliminated the precipitation of fibrinogen/fibrin in the kidney after reflow; dif, × 190. ***d***, Scanning electron microscopy of glomerulus of an untreated rat, showing network of fibres suggested to be fibrin and localized to the urinary space as in (*a*); freeze-drying, gold-palladium coating, × 435. ***e***, Experimental animal as in (*a*) and (*b*), showing in addition dense fibrillar deposits in the intertubular vessels and tubular basement membrane, dif × 190.

finally dried from CO_2 using a critical point drier; the specimens were then coated with a gold and palladium alloy. Some kidney pieces were cracked before freeze-drying, and then coated directly in order to eliminate all washing and fixation steps.

Transmission electron microscopy. Cubes measuring about 1 mm^3 were cut from the cortex and fixed in 2% glutaraldehyde in 0.1 M Na-cacodylate-HCl for 12 h, post-fixed in 1% osmium tetroxide for 2 h, dehydrated, and embedded in Epon. Sections, 1 μm thick, were cut, stained with Toluidine blue, and analysed for fibrin-like deposits. Positive glomeruli were cut for electron microscopy and post-stained in lead citrate and uranyl acetate.

Results

Immunohistochemistry

Immune reactive fibrinogen/fibrin could be detected in the glomeruli from 5 min after releasing the clamp, with a maximum at 15 min and decreasing after 60 min. Large amounts of fibrinogen/fibrin-related antigens were localized to the urinary space and between the capillary lobuli (Fig. 1*a*), but not convincingly in the capillary lumina. The deposits showed a dense fibrillary pattern, and the precipitates seemed to adhere to the visceral epithelial cells. No deposits could be shown in glomeruli, tubules, or vessels in the controls (Fig. 1*b*) or in the heparin-treated rats (Fig. 1*c*). The tubules showed a maximum of fibrinogen/fibrin deposits 15 min

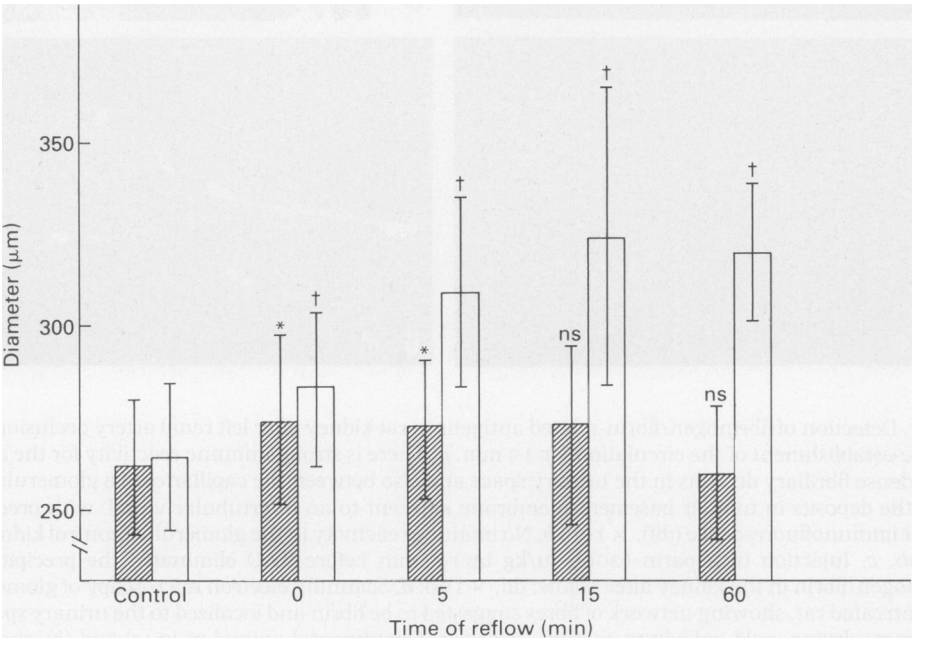

Fig. 2. Difference in diameter of the glomerular capillary tufts (▨) and whole glomeruli (□), respectively, in control and experimental animals after RAO and reflow (mean ± 1 s.d.). There was a statistically significant difference in whole glomerular diameter between controls and all experimental groups (†$P<0.001$). The diameter of the glomerular capillary tuft was larger during the first 5 min of reflow (*$P<0.05$), but subsequently the difference was not statistically significant (ns).

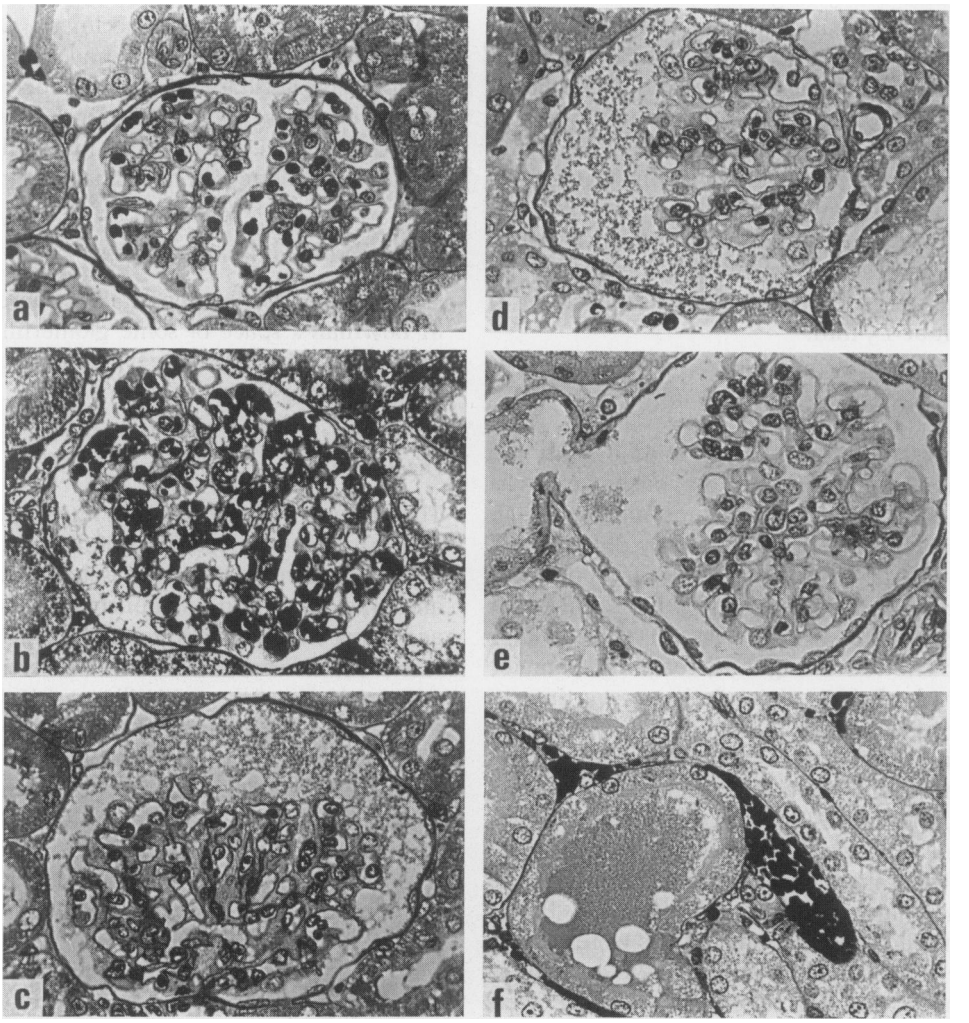

Fig. 3. Glomeruli from control and experimental kidneys. Formol–sublimate fixation, 1 μm sections cut with glass knife. PASM, × 360. ***a***, Control; ***b***, RAO, no reflow: the capillaries are engorged and the urinary space is slightly widened. ***c***, RAO and reflow for 5 min: the urinary space is now greatly dilated and filled with a granular precipitate. ***d***, RAO and reflow for 15 min: the urinary space is greatly dilated and contains precipitates; note that the glomerular capillary tuft is now of normal size. ***e***, RAO and reflow for 60 min : the glomerulus is still large, but the capillary tuft is not enlarged; the precipitates in the urinary space are much less prominent. ***f*** RAO and reflow for 60 min: dense granular casts are seen in the proximal tubules, which have stained positively with picro-Mallory, suggesting fibrinogen/fibrin related material; note also the engorged intertubular capillary vessels. PASM, × 360.

after releasing the clamp, and the deposits persisted as casts in the distal tubules at 60 min. Fairly large amounts of fibrinogen/fibrin-related antigens could also be detected in association with the tubular basal membrane. The vasa recta showed dense intravascular precipitates after 5 min, with a maximum at 15 min (Fig. 1*a,e*) and only small amounts could be demonstrated after 60 min. Albumin-related antigens could not be found in the glomeruli, but appeared in the tubules; they were largely localized to the zone of brush border, and were most abundant at 15 min.

Light microscopy

The results of the morphometric study are summarized in Fig. 2. Most conspicuous was the enlargement of the glomeruli (Figs 3*a–e*). This appeared to be caused by dilatation of the urinary space which began soon after release of the clamp and which reached a maximum at 15 min (Fig. 3*d*). The capillary tuft showed a slight increase in diameter already before recirculation, reverting to normal after 15 min (Fig. 3*d*). The initial increase in the capillary tuft diameter corresponded to distention of glomerular capillaries with blood (Fig. 3*b*). The urinary space showed a granular precipitate (Fig. 3*d,e*) which stained irregularly for fibrin with the picro-Mallory but not with the PTAH method. The same precipitates were seen in the tubules (Fig. 3*f*), where the casts were densely granular after 15 min. The vasa recta became engorged with blood immediately after releasing in the clamp, and remained distended after 60 min (Fig. 3*f*). The heparin-treated animals showed fewer deposits in the tubules and in the urinary space, which was not distended.

Scanning electron microscopy

The glomeruli were seen to be enlarged during the re-establishment of blood flow, and the urinary space contained a granular precipitate which was completely lacking in the glomeruli of control kidneys. Dense fibrillary deposits could be demonstrated in the glomerular urinary space at 15 min of reflow in the freeze-dried specimens, where no further incubation steps had been performed. This fibrillary precipitate showed the characteristics of fibrin fibres (Fig. 1*d*) with a network pattern. No fibrillary material was found in the kidneys of heparinized animals.

Transmission electron microscopy

Granular and fibrillar deposits were observed in Bowman's space of some glomeruli; the fibrillar deposits lacked the typical periodicity of polymerized fibrin.

Discussion

The present study shows that intracapillary and intratubular deposition of fibrin occurs in the rat kidney after renal ischaemia. The amounts of fibrin demonstrated varied according to the techniques used. The fibrin was most clearly visible in freeze sectioned preparations using antibodies against rat fibrinogen, whereas light microscopy of picro-Mallory stained immersion-fixed sections showed only small amounts of fibrin. No such reactivity was seen in the PTAH-stained sections, possibly because of loss of small amounts of loose fibrin deposits during dehydration of the sections. Fibrin may also be lost as a result of the fibrinolytic activity of the kidney before penetration of the fixation fluid. The specificity of the antibody reaction was strengthened by our use of species-specific anti-rat fibrinogen antibodies and the absence of cross reaction with rat albumin.

Loss of fibrin by rinsing during preparation or by fibrinolysis may thus explain failure to demonstrate fibrin by Mason *et al.* (1984) and Frega *et al.* (1976), who used immersion or perfusion fixation. With frozen material these errors can be avoided.

The fibrin was situated both intra- and extra-vascularly, which is an expression of different phenomena. Intravascular fibrin

deposition occurred in the peritubular but not convincingly in the glomerular capillaries. The coagulation could not have taken place during the period of occluded circulation, because fibrin was not present in kidneys examined before recirculation. However, the deposition of fibrin occurred very shortly thereafter, and was clearly detectable at 5 min, with a maximum at 15 min after RAO. What triggers the coagulation is not known, but capillary endothelial damage and platelet aggregation after re-establishment of the blood flow are possible mechanisms. The fibrin seen in the peritubular capillaries and vasa recta could have been formed in the glomerular capillaries and further embolized although we found no convincing reactivity for fibrinogen/fibrin in the glomerular capillaries. Increase in intracapillary haematocrit values in the inner stripe of the outer zone of the renal medulla (Karlberg 1982; Mason *et al.* 1984) with impaired medullary perfusion might be of importance for the formation or trapping of fibrin in this part of the renal vasculature.

Considerable amounts of fibrin-positive material were seen in Bowman's space in the fluorescent antibody prepared sections. This material appeared granular and fibrillar on electron microscopic examination. Similar material was also observed in the tubular lumina, sometimes condensed to tubular casts. The occurrence of this fibrin/fibrinogen material is apparently a result of filtration of various types of plasma proteins over the glomerular capillary wall, and precipitation of the fibrinogen. It is known that increased permeability to plasma proteins occurs after renal ischaemia (Racusen *et al.* 1982). Such leakage of proteins takes place despite a reduced glomerular filtration rate (Oken 1983). The material in the urinary space showed morphological characteristics of fully polymerized fibrin such as seen on scanning electron microscopy after freeze drying of the tissue. On transmission electron microscopy, however, only scanty material could be demonstrated, possibly owing to extraction by the filtration and dehydration steps.

The fibrin/fibrinogen material might constitute partly polymerized fibrin monomer complexes or fibrin partly degraded by fibrinolysis. Similar material has been reported in the glomeruli of patients with ARF (Clarkson *et al.* 1970; Vasalli & McCluskey 1964).

Tubular obstruction is a characteristic feature of the renal artery occlusion model of ARF (Solez 1983). Tubular cellular damage and swelling might be of importance for this obstruction, as could also the formation of casts from desquamated material from such cells. The casts formed by inspissation of the fibrinous material in the tubules observed in the present study might contribute to the obstruction of the tubular flow in this model. Our finding of increased size of the glomeruli owing to dilatation of Bowman's space shortly after re-establishment of the renal circulation is in accordance with such a mechanism. Whether the deposition of fibrin in the renal capillaries is of importance for the renal functional impairment of this model is an open question. Obstruction of the blood flow by fibrin is improbable, owing to the small size of the fibrin deposits. Liberation of vasoactive substances in connection with the coagulation process leading to vascular constriction or increased vascular permeability is however a possibility that cannot be excluded.

Acknowledgements

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